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Development of a Virosome Vaccine Against Avian Metapneumovirus Subtype C for Protection in Turkeys

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SUMMARY. An avian metapneumovirus (aMPV) virosome vaccine was prepared and tested for protection of turkeys by aMPV challenge. The vaccine was produced using a detergent-based (Triton X-100) extraction of aMPV subtype C followed by detergent removal with SM2 Bio-Beads. Western blot and virus-neutralization analysis confirmed that the aMPV virosomes contained both the fusion and attachment glycoproteins. Specific-pathogen-free turkeys were immunized either intranasally (IN) or intramuscularly (IM) with two doses of the aMPV virosome vaccine. Vaccination decreased clinical signs of disease following virulent challenge, and IN vaccination was superior to IM vaccination in reducing clinical signs. Decreases in viral load in the respiratory tract were observed in turkeys receiving IN vaccination with aMPV virosomes compared to unvaccinated poults. Increased virus-neutralizing antibody levels against aMPV were observed in birds vaccinated with virosomes. These results demonstrate that immunization of turkeys with aMPV virosomes can be an effective strategy for control of disease.

RESUMEN. Desarrollo de una vacuna con virosomas para la protección contra metapneumovirus aviares.

Se desarrolló una vacuna con virosomas para metapneumovirus aviares y se estudió la protección en pavos mediante el desafío. La vacuna fue preparada mediante la extracción de metapneumovirus con detergente (Tritón X-100), seguida por la remoción con perlas Bio-Beads SM2. Los análisis de inmunotransferencia y de virus neutralización confirmaron que los virosomas de metapmeumovirus contenían las glicoproteínas de fusión y de adherencia. Se inmunizaron aves libres de patógenos específicos por las vías intranasal o intramuscular con dos dosis de vacunas con virosomas contra metapneumovirus. La vacunación disminuyó los signos clínicos de la enfermedad ante el desafío con virus virulento. La vacunación intranasal fue superior para reducir los signos clínicos en comparación con la vacunación intramuscular. Se observó disminución en la carga viral del aparato respiratorio en los pavos que recibieron la vacunación intranasal con virosomas en comparación con los pavos no vacunados. Se observó incremento en los niveles de anticuerpos neutralizantes contra metapneumovirus en los pavipollos vacunados con virosomas. Estos resultados demuestran que la inmunización de los pavos con virosomas puede ser una estrategia efectiva para el control de la enfermedad.

Key words: avian metapneumovirus, veterinary virology, virosome vaccine, turkeys, immunology, turkey rhinotracheitis virus

Abbreviations: aMPV = avian metapneumovirus; CPE = cytopathic effect; ELISA = enzyme-linked immunosorbent assay; F = fusion; G = attachment; Ig = immunoglobulin; IM = intramuscular; IN = intranasal; L = RNA-dependent RNA-polymerase; MA = maternal antibody; Mn = Minnesota; N = nucleocapsid; NC = nonchallenged; NDV = Newcastle disease virus; OD = optical densities; P = phosphoprotein; PBS = phosphate-buffered saline, pH 7.2; PBS-T = PBS containing Tween 20; PC = postchallenge; RSV = respiratory syncytial virus; RT-PCR = reverse transcription–polymerase chain reaction; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SPF = specific-pathogen-free; $TCID_{50}$ = 50% tissue-culture infectious dose; VN = virus neutralization

Avian metapneumovirus (aMPV) is the etiologic agent of turkey rhinotracheitis and a swollen head syndrome of turkeys and chickens, respectively. In the United States, outbreaks of aMPV were first identified during 1997 from turkeys in Colorado and, subsequently, in Minnesota. This recently emerged pneumovirus, part of the paramyxovirus family, causes a highly contagious respiratory tract disease. aMPV causes productivity losses rather than producing high mortality. The problem is still regional, primarily infecting turkeys in the state of Minnesota. No infections have been detected among turkeys in other intensive production areas or among chickens in any state, including Minnesota.

Currently only one serotype of aMPV has been described; however, nucleotide sequence analysis has identified four subtypes: A, B, and D—comprising European isolates—and subtype C, from U.S. isolates (6,16,41,42).

Historically, inactivated vaccines produced against viruses included within the pneumovirus subfamily, including human respiratory syncytial virus (RSV), bovine RSV, and parainfluenza virus, have either failed to protect or have enhanced lung pathology upon reinfection (8,20,27,34,40). One problem associated with parenteral administration of these vaccines is their inability to induce an effective mucosal immune response following natural infection. Inactivated or subunit vaccines delivered to mucosal surfaces are, in general, poor inducers of mucosal immunoglobulin (Ig) A.

In European countries, cell-culture–attenuated or inactivated vaccines are currently being used to control disease caused by subtypes A and B (15). However, at least one report indicates that attenuated live aMPV vaccines may promote the incidence of disease in young birds (28). Maternal antibodies (MA) against aMPV do not appear to protect poults against disease (29).

In the United States, inactivated vaccines against aMPV subtype C have not been shown to be protective (17,43). Likewise, previously published data examining an experimental attenuated-live aMPV vaccine indicated that the dose required for protection can exceed that required for infection (14).

Virosome vaccines are designed to contain viral membrane proteins within a liposome complex, yet they are noninfectious because they do not possess genetic nucleic acid. Virosome vaccines are advantageous because they are as safe as inactivated vaccines but behave as live virus and are able to attach and fuse with host cells. We have previously shown that virosomes can induce protection against lethal Newcastle disease virus (NDV) in chickens (18). Virosome vaccines have been developed against numerous viruses (10,13,19,22,25,33). In the present study, an aMPV virosome vaccine was developed and shown to be capable of protecting poults against clinical signs of disease and inducing antibody responses in turkeys following aMPV challenge. Moreover, the vaccine decreased viral load from respiratory samples following intranasal (IN) application.

MATERIALS AND METHODS

Virus. The Minnesota (Mn) 1A isolate of aMPV was used in these studies. The virus was isolated in 1997 from a turkey flock infected with aMPV in Minnesota. For virosome preparation, the virus was propagated in monolayers of Vero cells grown at 37 C in 5% CO_2 in F12/DMEM with 5% fetal bovine serum and antibiotics (Invitrogen, Baltimore, MD). For vaccine-challenge studies in Experiments II and III (below), the virus was passed three times in 11–14 day-of-embryonation specific-pathogen-free (SPF) turkey eggs and harvested from allantoic fluid.

Experimental animals. One-day-old SPF Beltsville White turkey poults were obtained from our flock at the Southeast Poultry Research Laboratory and used in these studies. Birds were housed in Horsfall isolation units under negative pressure in a biosafety level 3 agriculture facility (5). Birds received feed and water *ad libitum.*

Preparation of virosomes. aMPV virosomes were produced as previously described for NDV (18). Briefly, sucrose gradient purified Mn virus was resuspended in phosphate-buffered saline (PBS) (pH 7.2) at a protein concentration of 5 mg/ml. Triton X-100 (Fisher Scientific, Atlanta, GA) was added to a final concentration of 2% (v/v) and incubated at room temperature for 1 hr with gentle shaking. The suspension was centrifuged for 1 hr at 100,000 $\times$ *g* to remove nucleocapsid complexes. Detergent was removed by the stepwise addition of methanol-washed SM2 Bio-Beads (BioRad Laboratories, Inc., Hercules, CA) (12,23). One hundred sixty milligrams of SM2 Bio-Beads was added to 2 ml of supernatant, gently mixed by inversion, and allowed to incubate for 2 hr at room temperature; this was followed by the addition of 320 mg of SM2 Bio-Beads for an additional 2 hr at 4 C and an additional 640 mg overnight at 4 C. The reconstituted aMPV virosomes were collected with a needle and syringe to exclude the SM2 Bio-Beads. To ensure vaccine inactivation, reconstituted virosomes (diluted 1:10 in PBS) were tested by inoculation in Vero cells. Cells were monitored for cytopathic effect (CPE) by three successive passages. All virosome vaccine preparations were negative for CPE. Protein concen-

tration was determined colormetrically (Pierce, Rockford, IL).

Experimental design. Three vaccine-challenge experiments were performed using the aMPV virosome vaccine. In Experiment I, 22 one-day-old SPF turkeys were randomly divided into two groups of 11 birds each. Birds in group 1 received 100 μl of PBS via IN route at 3 and 4 wk of age. Birds in group 2 received 100 μl of aMPV virosomes (10 μg per bird) via IN route at 3 and 4 wk of age. One week after the second vaccination (5 wk of age), birds in each group were challenged via the IN route with 1×10^5 fifty percent tissue culture infective doses ($TCID_{50}$) Mn virus per bird. Three or four birds from each group were euthanatized at 2, 7, and 14 days postchallenge (pc) by overdose of sodium pentobarbital. Clinical signs were recorded for each bird prior to euthanatization. Serum was collected and tested for antibodies to aMPV by enzyme-linked immunosorbent assay (ELISA), as described below. At necropsy, lung wash (respiratory lavage) samples were collected for virus isolation as described below.

In Experiment II, 60 SPF turkey poults were randomly divided into four groups of 15 birds each. Birds in groups 1 and 2 received 100 μl of PBS (pH 7.4) via IN route at 7 and 21 days of age. Birds in group 3 received vaccination with 10 and 20 μg of aMPV virosomes via IN route on days 7 and 21 of age, respectively. Birds in group 4 received vaccination by intramuscular (IM) injection in the thigh region with 10 and 20 μg of aMPV virosomes on days 7 and 21 of age, respectively. Birds in groups 2, 3, and 4 were challenged with 1×10^6 $TCID_{50}$ Mn virus per bird 2 wk after last vaccination via IN route (day 35). On days 0, 3, 7, 11, and 14 pc, birds were scored for clinical signs. Serum was collected for aMPV antibody detection by ELISA, as described below.

In Experiment III, 27 one-day-old SPF turkeys were randomly divided into three groups of nine birds each. Poults in group 1 received PBS via IN routes on days 7 and 21 of age. Turkeys in groups 2 received 10 μg of aMPV virosomes at 7 and 21 days of age via IM route. Turkeys in group 3 received 10 μg of aMPV virosomes at 7 and 21 days of age via IN route. Birds were challenged at 35 days of age with 1×10^6 $TCID_{50}$ Mn virus per bird via IN route. Birds were monitored daily for clinical signs of disease and serum harvested for antibody testing by ELISA and virus neutralization (VN).

Respiratory lavage. Virus isolation from respiratory surfaces was performed using respiratory lavage as previously described (9). Briefly, following euthanatization, an incision was made from the mandible to the thoracic inlet. A catheter (3½ French, Soverign, manufactured by Sherwood Medical, St. Louis, MO) cut to 1.5 inches was attached to a three-way stopcock (VWR, Atlanta, GA) with an empty 50-ml syringe and a 10-ml syringe containing 10 ml PBS. The catheter was placed into the trachea to form an airtight seal, and a vacuum was formed by withdrawing air from the respiratory tract with the 50-ml syringe. The stopcock was turned to maintain vacuum and the PBS slowly injected into the trachea. After gently rocking the body from side to side, the PBS was withdrawn back into the 10-ml syringe. The respiratory lavage was repeated two to three times per bird using the same PBS. Fresh PBS was used for each individual bird. Lavage fluid was centrifuged ($3000 \times g$) to pellet debris and the supernatant harvested and stored at −20 C until use.

ELISA. Two separate ELISA assays were performed to measure antibody levels.

For Experiments I and II. Anti-aMPV antibodies were determined by ELISA, as previously described, with minor alterations (11). Alternating rows of microtiter plates (Immulon 4; Dynex, Chantilly, VA) were coated with 500 ng/well of inactivated aMPV in carbonate coating buffer (pH 9.6) for 3 hr at room temperature and held overnight at 4 C. Wells were washed three times with PBS containing 0.05% Tween 20 (PBS-T) (Sigma Chemical Co., St. Louis, MO) and incubated with 1% bovine serum albumin (Sigma Chemical Co.) in PBS-T for 1 hr at room temperature. Turkey serum (1:500) was diluted (as indicated) in PBS containing 1% bovine serum albumin and individually tested in triplicate wells for 1 hr at room temperature. Following three washes with PBS-T, goat anti-turkey IgG conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc., Birmingham, AL) was added to each well, incubated for 1 hr, and washed three times with PBS-T. Substrate (SigmaFast OPD tablet set, Sigma Chemical Co.) was added to each well according to the manufacturers' directions. Recorded optical densities (ODs) were determined by subtracting mock-coated wells from aMPV-Mn virus–coated wells.

For Experiment III. Microtiter plates were coated as above and serial two-fold dilutions of serum in PBS-T were added to individual wells and incubated for 1 hr at room temperature. Wells were washed and substrate added as described above. Endpoint titrations were performed from negative control turkey sera. Results are expressed as mean reciprocal dilution of serum giving OD greater than mean OD plus three standard deviations of the negative control sera.

Western blot. Purified aMPV proteins from whole virus and virosome preparations were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% polyacrylamide gel using the Bio-Rad Criterion system (21). Following transfer onto nitrocellulose membranes (Bio-Rad), aMPV proteins were reacted with mouse anti-aMPV antisera as previously described (18).

Virus neutralization. Sera were tested for virus neutralization of aMPV using Vero cells grown in 96-well, flat-bottomed microtiter plates (Costar; Corning, Ithaca, NY), as previously described (11). All samples

were diluted two-fold, incubated with 100 $TCID_{50}$ Mn virus, and tested in triplicate. Positive and negative serum controls were added to each group of plates tested. Vero cells were monitored for cytopathic effect (CPE) for 7 days postinoculation. The VN titer was expressed as the reciprocal of the highest dilution that completely inhibited cytopathic effect. The mean VN titer per group was determined by adding the VN titers of a group of birds and dividing it by the total number of birds.

Virus isolation. Respiratory lavage fluid from individual birds were diluted two-fold and placed on Vero cell monolayers in media containing antibiotics. Samples were adsorbed for 1 hr at 37 C and replaced with fresh media. The Vero cells were allowed to grow for 5 days, as described above. Typical aMPV syncytia formation on Vero cells indicated a positive aMPV isolation, which was confirmed by reverse transcription–polymerase chain reaction (RT-PCR). All samples were passaged individually up to five times. If no syncytia formation was observed, the sample was considered negative for aMPV. The supernatants from the last passage of all samples were tested for the presence of aMPV by RT-PCR as described below.

Virus detection with RT-PCR. For RT-PCR, RNA was extracted from homogenized tissues with an RNA extraction kit (RNeasy™; Qiagen, Valencia, CA) according to manufacturer's directions. Primers were designed to the F gene using known sequences (GenBank accession no. AF187153) with OLIGO™ version 4.0 software (National Bioscience, Plymouth, MN). The forward primer sequence was 5′-GACAAGTGAAAATGTCTT-3′ and the reverse primer sequence 5′-AACTAAAATTAAGGGATA-3′, with a predicted product of 1633 base pairs. The RT was performed with the SuperScript II RT-PCR kit (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's recommendations. Three microliters of extracted RNA was used to make cDNA with the forward primer in a reaction volume of 20 µl. Following first strand production, PCR was performed using 3 µl of cDNA, 20 pmol of forward and reverse primer, and additional PCR reagents in a 50-µl reaction volume. Following denaturation at 94 C for 2 min, 35 cycles of denaturation, annealing, and extension at 94 C for 30 sec, 53 C for 1 min, and 72 C for 2 min, respectively, were performed. A final extension was completed at 72 C for 10 min. The products were analyzed by electrophoresis on an 0.8% agarose gel, stained with ethidium bromide, and visualized with an ultraviolet transilluminator. Positive and negative controls consisting of RNA extracted from aMPV- and mock-infected Vero cells, respectively, were included for each group of reactions.

RESULTS

A detergent-based (Triton X-100) procedure was employed to isolate the membrane-bound proteins (e.g., fusion [F] and attachment [G]) and to remove them from the detergent insoluble proteins (e.g., RNA-dependent RNA-polymerase [L], phosphoprotein [P], or nucleoprotein [N]). Using SDS-PAGE analysis, two major protein bands with approximate molecular weights of 58 and 78 kD were identified. When tested by western blot analysis, using polyclonal mouse anti-aMPV antisera, both proteins react positively (Fig. 1a). Based on extraction procedure and predicted molecular weight, the proteins are presumably the F and G glycoproteins. In some preparations, a minor reaction was also observed with an approximate 40-kD protein, which presumably is the matrix protein (data not shown). To further confirm that the virosomes contained the proteins responsible for VN activity, a high-titered virus-neutralizing anti-aMPV turkey antisera was preincubated with two-fold dilutions of virosomes prior to testing for VN activity. As presented in Fig. 1b, neutralizing titer of the antisera was reduced by preincubation with virosomes. These results indicate that the epitopes necessary for inducing VN antibodies were contained within the virosome preps. To confirm that the replication complex (L,N,P) had been removed from the vaccine, the virosomes were incubated on Vero cells for 5–7 days and passaged onto fresh cells. No CPE was observed following five passages, indicating the virosomes were noninfectious (data not shown).

Experiment I. Following IN virosome vaccination, SPF turkeys were challenged with 1×10^5 $TCID_{50}$ Mn virus per bird. No clinical signs of disease were observed in either the vaccinated or challenge-control group (data not shown). Therefore, protective immunity could not be established based on clinical signs. However, differences in viral load from respiratory lung washes were observed. Turkeys vaccinated with virosomes had decreased virus titers in respiratory tissues compared with controls, but vaccination was not able to completely clear the virus from these birds (Fig. 2). Because of the lack of clinical signs observed in birds during Experiment I, the challenge dose used in subsequent experiments was increased to 1×10^6 $TCID_{50}$ per bird.

Individual serum samples were collected and tested for the presence of ELISA titers against aMPV Mn virus. Although vaccination did not appear to increase serum antibody levels prior to challenge, vaccinated poults exhibited increased serum IgG antibodies compared to control birds following challenge on 7 and 14 days PC (Fig. 3).

Experiment II. Turkey poults received two doses of virosome vaccine via IN or IM route.

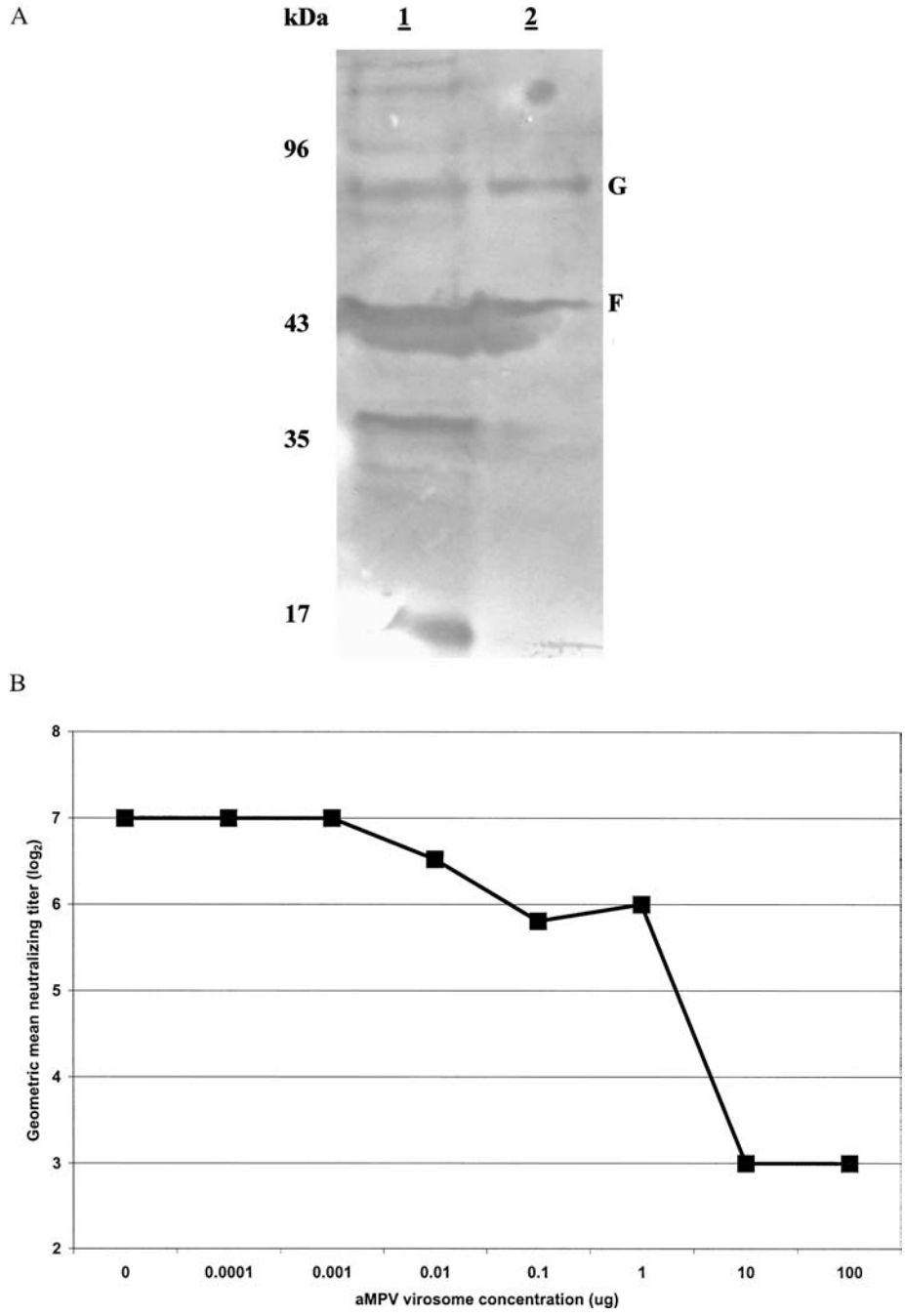


Fig. 1. (A) Biochemical analysis of aMPV virosomes. Western blot using mouse anti-aMPV polyclonal antisera of aMPV Mn virus (Lane 1) and aMPV virosomes (Lane 2) demonstrating F and G glycoproteins contained in vaccine. Molecular weight standards (kD) are indicated on the left. (B) Preincubation with aMPV virosomes decreases the neutralizing titer of turkey sera. Turkey sera of known neutralizing titer (1:128) was preincubated for 1 hr at room temperature with decreasing concentrations of virosomes. The turkey sera was then tested for neutralizing activity against 100 $TCID_{50}$ Mn virus. Geometric mean titer determined as highest dilution of sera that completely inhibited cytopathic effect.

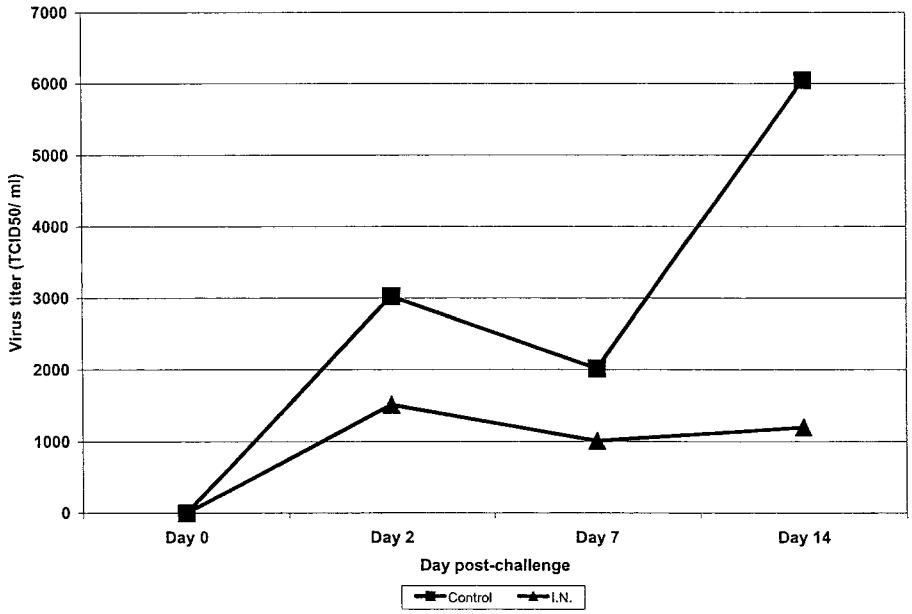


Fig. 2. aMPV viral load following intranasal (IN) virosome vaccination in Experiment I. Control and vaccinated turkeys were challenged via IN route with 1×10^5 $TCID_{50}$ Mn virus. Following euthanatization, respiratory lavage fluid was tested for infectious virus by passage on Vero cells. All samples were passaged five times, tested in triplicate, and confirmed by RT-PCR. Results are expressed as geometric mean 50% TCID/ml fluid.

Decreases in clinical signs were observed in both groups of birds receiving virosomes (Fig. 4a). Clinical signs included nasal exudate, snicking, or frothy ocular discharge. Birds vaccinated via IN route displayed fewer clinical signs than did birds receiving an IM route of vaccination. The duration of clinical signs was also decreased in birds receiving virosomes via IN route as compared to birds in other groups. At 7 days PC, 100% of control birds were exhibiting clinical signs of disease, which was mainly observed as nasal exudate and snicking. In comparison, 86% and 29% of IM or IN vaccinated birds, respectively, displayed clinical signs on day 7 PC. As seen in Fig. 4b, poults vaccinated by either route displayed higher serum IgG antibody from day 7 PC. The increased antibody levels appeared to correlate with the level of protection against clinical signs of disease, although little aMPV-specific IgG antibody was detected prior to challenge in vaccinated birds.

Experiment III. Turkeys were vaccinated via IN or IM route, as described in Experiment II, but with different doses of virosomes. Protection from clinical signs of disease was again reduced in birds receiving virosome vaccination (Fig. 5). Control-challenged birds displayed clinical signs between days 5 and 11 PC. On days 6 and 7 PC, 66% of birds displayed signs. In contrast, only 11% of birds receiving IN virosome vaccination displayed clinical signs following challenge, which were only observed for 3 days (day 6 through 8 PC). In the IM-vaccinated group, 33% of birds displayed clinical signs on day 8 PC. Clinical signs included nasal exudate, snicking, or frothy ocular discharge. Both the duration and number of birds showing clinical signs of disease were reduced by virosome vaccination.

ELISA titers were determined from differences in OD with negative control sera. Unvaccinated birds did not exhibit increases in either ELISA or VN titers until day 14 PC (Table 1). In contrast, IM-vaccinated birds rapidly increased both ELISA and VN titers by a sixfold measure on day 7 PC, compared to day 0. By day 14 PC, titers had slightly reduced in IM-vaccinated birds, compared to results on day 7 PC. For IN-vaccinated turkeys, high ELISA values were observed on day 0, which did not correspond to high VN titers. However, by days 7 and 14 PC, serum VN titers had increased 40- and 15-fold over day 0 values, respectively.

DISCUSSION

The recent emergence of aMPV into domestic turkeys in the central United States emphasizes the

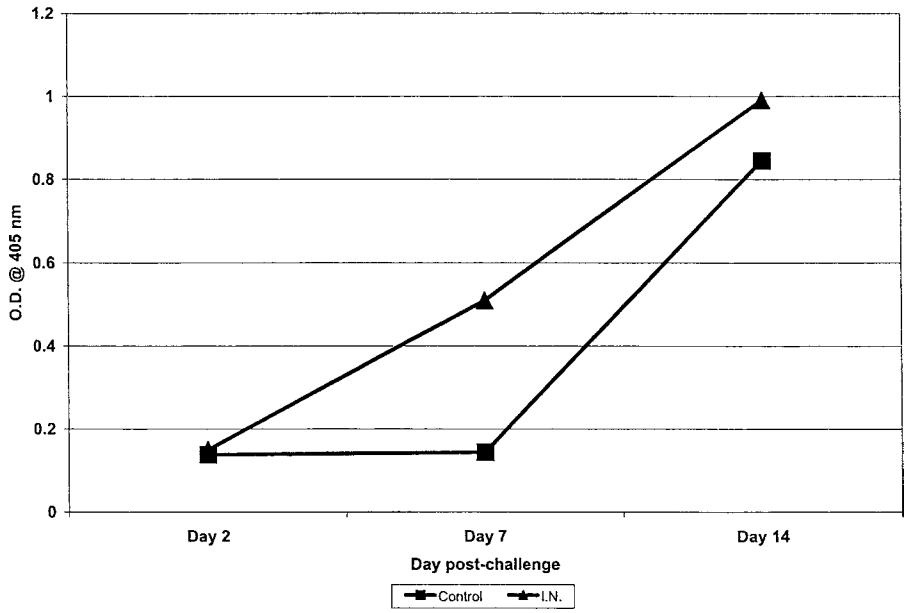


Fig. 3. Serum IgG immune response to aMPV following PBS (control) or intranasal (IN) immunization with virosomes in Experiment I. Poults were challenged with 1×10^5 $TCID_{50}$ Mn virus by IN route. Serum IgG was detected following a 1:500 dilution tested in triplicate by ELISA. Results are expressed as mean experimental OD minus background OD.

importance of production of efficacious vaccines to control infection and disease. Because the virus replicates in respiratory tissues, mucosal immunity is a crucial factor when developing an effective vaccine formulation. The difficulty in stimulating mucosal immunity from inactivated or subunit vaccines has resulted in research aimed at improving delivery of mucosal antigens.

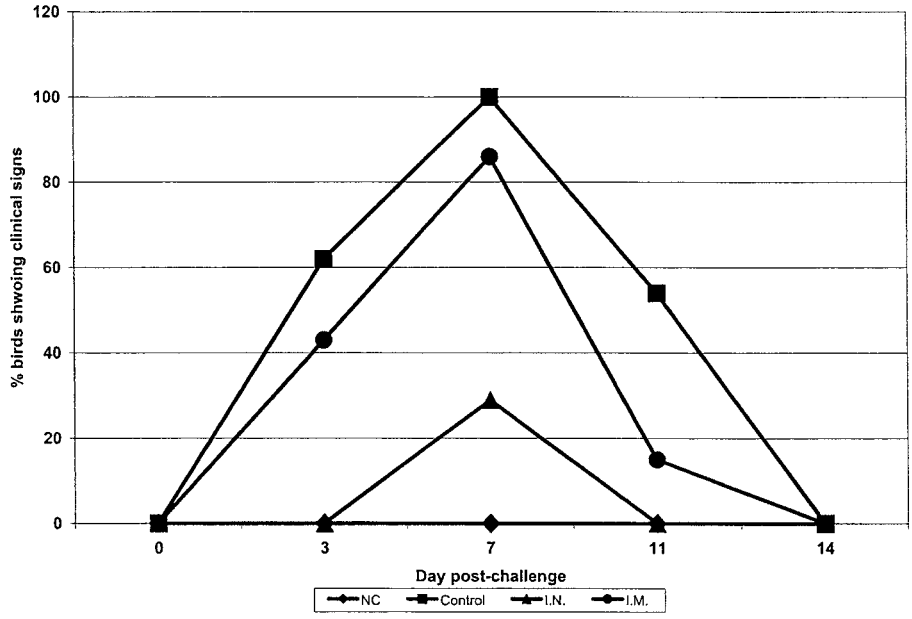
In the present study, using a detergent-based extraction procedure, a virosome vaccine was constructed using aMPV that contained the F and G glycoproteins. The F and G glycoproteins of aMPV are membrane bound and contain epitopes that induce VN antibodies that will protect birds from disease. Functional analysis indicated that the epitopes necessary for virus neutralization were contained within the virosomes, since pretreatment of aMPV immune sera decreased virus-neutralizing titer. Vaccination of turkeys with aMPV virosomes provided increased protection from challenge, whereas nonvaccinated birds developed more clinical signs that persisted longer. Vaccination of turkeys with aMPV virosomes increased VN immune response following aMPV challenge in these studies. In addition, virosome-vaccinated birds exhibited decreases in virus titer from respiratory surfaces.

We have previously shown that NDV virosomes containing functionally active fusion and hemagglutinin–neuraminidase proteins can induce protection against lethal challenge in chickens (18). Other reports have detailed the production of virosomes from enveloped viruses for studies involving fusion proteins, gene transfer, and vaccination (4,7,13,25, 32,33,35,36). This report is the first to describe protection following vaccination with aMPV virosomes.

Comparison of the antibody responses following virosome vaccination resulted in increases of both ELISA and VN antibody titers following challenge. An association between the presence of ELISA or VN antibodies and protection from challenge was observed during Experiments II and III. Previous research with virosomes of influenza, hepatitis virus, and respiratory syncytial virus demonstrated that both intranasal (10,24) and intramuscular (39) routes of vaccination increased humoral immune responses, resulting in protection from challenge. In this study, two doses of virosomes were given per bird within a 2-wk interval. Whether or not two vaccinations is a requirement for protective immunity is the subject of ongoing studies.

Development of inactivated vaccines produced against other viruses in the *Pneumovirinae* subfamily

A



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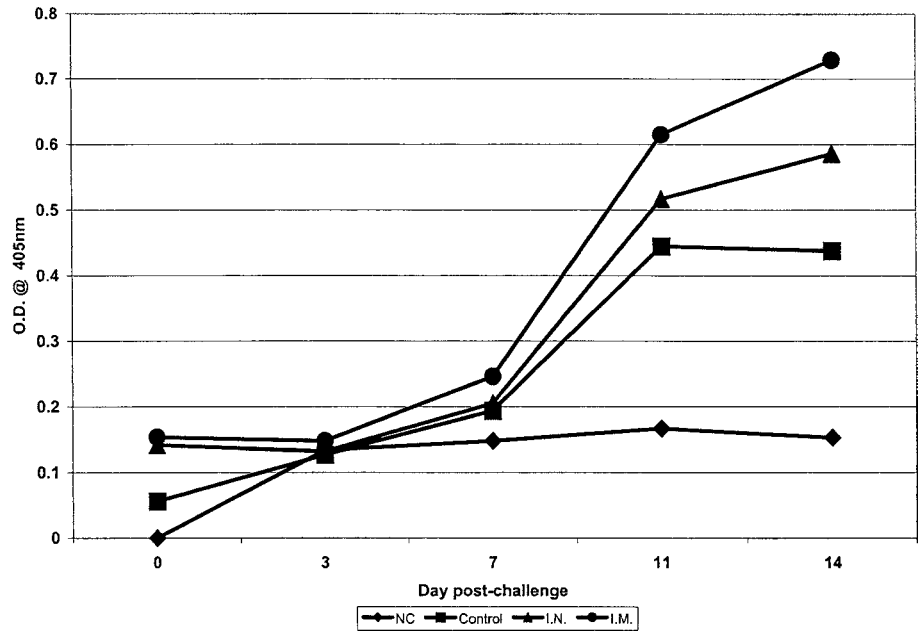

Fig. 4. (A) Percent birds exhibiting clinical signs after challenge with aMPV and (B) serum ELISA results from Experiment II. Poults were vaccinated twice with either PBS (control) or aMPV virosomes via intranasal (IN) or intramuscular (IM) route. The birds were challenged with 1×10^6 $TCID_{50}$ Mn virus 2 wk after secondary vaccination and scored for clinical signs of disease at 0, 3, 7, 11, and 14 days PC. Sera were diluted 1:500 and tested for IgG antibodies against aMPV. Results are expressed as mean experimental OD minus background OD. NC = Nonchallenged poults.

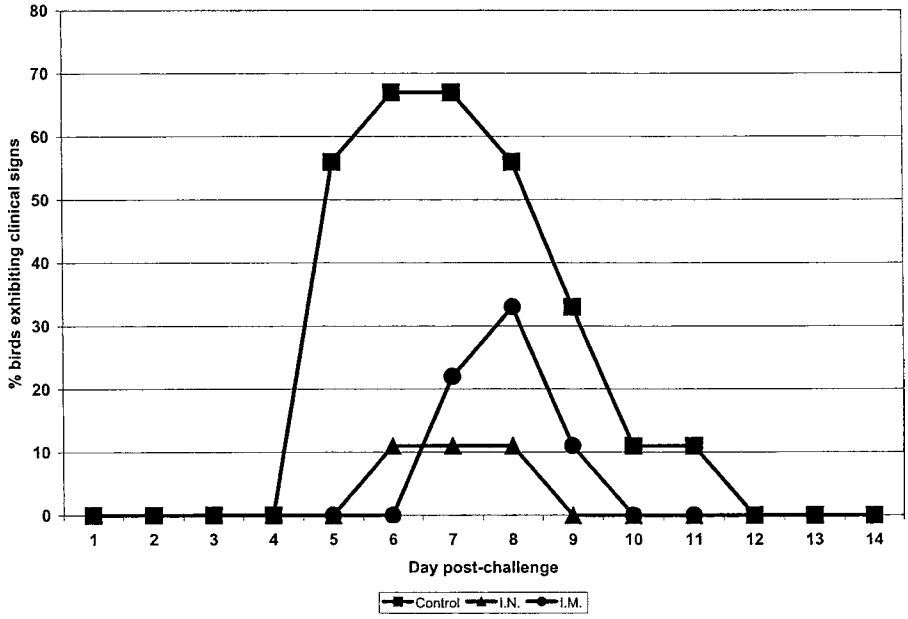

Fig. 5. Percent birds exhibiting clinical signs after challenge with aMPV in Experiment III. Poults were vaccinated twice with either PBS (control) or aMPV virosomes via intranasal (IN) or intramuscular (IM) route. The birds were challenged with 1×10^6 $TCID_{50}$ Mn virus 2 wk after secondary vaccination and examined for clinical signs of disease daily.

have not always proven advantageous; in fact, an enhancement of disease following natural infection as been described in the literature (8,20,27,34,40). It was later established that inactivation of human RSV disrupts epitopes on the F and G surface proteins necessary to illicit neutralizing antibodies (26). This inactivation phenomenon has also been described with other members of the *Paramyxovirus* family, as well vesicular stomatitis virus (3,30,31). These observations may also explain why MA do not protect poults against virus challenge (29). In those studies, poults with high MA levels, based on ELISA, were not protected from virus challenge. However, since the dams of the progeny birds used in the experiment were vaccinated with a killed aMPV vaccine 6 wk prior, it may be that antibodies produced were to nonneutralizing epitopes and were thus nonfunctional. The VN capability of antibodies from those birds was not reported.

Another advantage of virosome vaccines are their nonreplicating properties. Virosomes do not contain viral replication proteins (e.g., L, N, or P). Serologic testing to these proteins can identify poults that have been exposed to field virus, since virosome-vaccinated birds will not produce antibodies against these antigens. Use of either live-virus or inactivated-virus vaccines would not permit this type of differentiation, since vaccinated birds would have antibodies to all aMPV proteins.

Virosomes have been shown to stimulate both

Table 1. Serum antibody response following aMPV challenge of turkeys in Experiment III, as determined by ELISA and VN response.

Group	Day 0	Day 7	Day 14
Control			
ELISA[A]	2	2	5
VN[B]	2	2	11
Intramuscular			
ELISA	2	13	8
VN	2	13	9
Intranasal			
ELISA	15	12	47
VN	1	40	15

[A]ELISA titers expressed as the mean reciprocal dilution of serum yielding an optical density greater than the mean negative control plus 3 standard deviations.

[B]VN titer expressed as mean of the reciprocal of the highest dilution that completely inhibited cytopathic effect.

MHC type I and II activation, inducing cellular and humoral immune responses, respectively (1,2,38). Internalization of aMPV antigens may provide for MHC class I and II presentation in turkey poults and stimulate virus-specific cytotoxic T cells. Cytotoxic T-cell responses were stimulated following virosome delivery of influenza (13) and Ebola virus antigen (37,38). Whether aMPV virosomes stimulate cytotoxic T cells is the subject of ongoing studies.

This research provides useful information about aMPV vaccination. The use of virosomes to deliver aMPV antigens stimulated increased protective immunity against challenge. Since respiratory tract infections constitute a significant amount of morbidity and mortality, and therefore cost, to the poultry industry, protection against infection at mucosal sites is an important aspect of vaccine design. Virosomes delivered to respiratory tissues decreased both clinical signs of disease and virus load. Thus, stimulation of local immunity was provided in a nonreplicating vaccine formulation. This technique for vaccine production may prove useful for other enveloped avian viruses and has potential for intracellular delivery of macromolecules *in vivo* or *in ovo*. Future development, including the construction of bi- and trivalent virosome formulations to include protein antigens from other viral pathogens may make this type of vaccine an attractive alternative to inactivated or live vaccines.

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